

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68		A2	(11) International Publication Number: WO 99/31269
			(43) International Publication Date: 24 June 1999 (24.06.99)
(21) International Application Number: PCT/CA98/01151		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 10 December 1998 (10.12.98)			
(30) Priority Data: 2,218,446 12 December 1997 (12.12.97) CA			
(71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): BUSSEY, Howard [CA/CA]; 325 Victoria, Westmount, Québec H3Z 2N1 (CA). LUSSIER, Marc [CA/CA]; 7790 St-Gérard, Montréal, Québec H2R 2K4 (CA). SDICU, Anne-Marie [CA/CA]; 12359 Granger, Pierrefonds, Québec H8Z 1V4 (CA). SHAHINIAN, Sarkis, Serge [CA/CA]; 5027 Christophe-Colomb, Montréal, Québec H2J 3H1 (CA).		Published Without international search report and to be republished upon receipt of that report.	
(74) Agents: CÔTÉ, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).			

(54) Title: NEW CANDIDA ALBICANS KRE9 AND USES THEREOF

(57) Abstract

The present invention relates to an isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein the gene is referred to as *CaKRE9*, wherein the sequence of the DNA is as set forth in Fig. 1. The present invention relates to antifungal *in vitro* and *in vivo* screening assays for identifying compounds which inhibit the synthesis, assembly and/or regulation of β 1,6-glucan. There is also disclosed an *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		

NEW CANDIDA ALBICANS KRE9 AND USES THEREOFBACKGROUND OF THE INVENTION(a) Field of the Invention

5 The invention relates to a novel gene, CaKRE9, isolated in the yeast pathogen, *Candida albicans*, that is a functional homolog of the *S. cerevisiae* KRE9 gene and which is essential for cell wall glucan synthesis, and to novel antifungal screening assays.

(b) Description of Prior Art

Fungi constitute a vital part of our ecosystem but once they penetrate the human body and start spreading they cause infections or "mycosis" and they can pose a serious threat to human health. Fungal infections have dramatically increased in the last 2 decades with the development of more sophisticated medical interventions and are becoming a significant cause of morbidity and mortality. Infections due to pathogenic fungi are frequently acquired by debilitated patients with depressed cell-mediated immunity such as those with human immunodeficiency virus (HIV) and now also constitute a common complication of many medical and surgical therapies. Risk factors that predispose individuals to the development of mycosis include neutropenia, use of immunosuppressive agents at the time of organ transplants, intensive chemotherapy and irradiation for hematopoietic malignancies or solid tumors, use of corticosteroids, extensive surgery and prosthetic devices, indwelling venous catheters, hyperalimentation and intravenous drug use, and when the delicate balance of the normal flora is altered through antimicrobial therapy.

The yeast genus *Candida* constitutes one of the major groups that cause systemic fungal infections and 35 the five medically relevant species which are most

often recovered from patients are *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*.

Much of the structure of fungal and animal cells along with their physiology and metabolism is highly conserved. This conservation in cellular function has made it difficult to find agents that selectively discriminate between pathogenic fungi and their human hosts, in the way that antibiotics do between bacteria and man. Because of this, the common antifungal drugs, like amphotericin B and the azole-based compounds are often of limited efficacy and are frequently highly toxic. In spite of these drawbacks, early initiation of antifungal therapy is crucial in increasing the survival rate of patients with disseminated candidiasis. Moreover, resistance to antifungal drugs is becoming more and more prominent. For example, 6 years after the introduction of fluconazole, an alarming proportion of *Candida* strains isolated from infected patients have been found to be resistant to this drug and this is especially the case with vaginal infections. There is thus, a real and urgent need for specific antifungal drugs to treat mycosis.

The fungal cell wall: a resource for new antifungal targets

In recent years, we have focused our attention on the fungal extracellular matrix, where the cell wall constitutes an essential, fungi-specific organelle that is absent from human/mammalian cells, and hence offers an excellent potential target for specific antifungal antibiotics. The cell wall of fungi is essential not only in maintaining the osmotic integrity of the fungal cell but also in cell growth, division and morphology. The cell wall contains a range of polysaccharide polymers, including chitin, β -glucans and O- and N-linked mannose sidechains of glycoproteins. β -glucans, homopolymers of glucose, are the main structural component

of the yeast cell wall, and constitute up to 60% of the dry weight of the cell wall. Based on their chemical linkage, two different types of polymers can be found: β 1,3-glucan and β 1,6-glucan. The β 1,3-glucan is the 5 most abundant component of the cell wall and it contains on average 1500 glucose residues per molecule. It is mainly a linear molecule but contains some 1,6-linked branchpoints. The β 1,6-glucan is a smaller and highly branched molecule comprised largely of 1,6-linked glucose residues with a small proportion of 1,3-linked residues. The average size of β 1,6-glucan is approximately 400 residues per molecule. The β 1,6-glucan polymer is essential for cell viability as it acts 10 as the "glue" covalently linking glycoproteins and the 15 cell wall polymers β 1,3-glucan and chitin together in a crosslinked extracellular matrix.

It would be highly desirable to be provided with the identification and subsequent validation of new cell wall related targets that can be used in specific 20 enzymatic and cellular assays leading to the discovery of new clinically useful antifungal compounds.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide 25 the identification and subsequent validation of a new target that can be used in specific enzymatic and cellular assays leading to the discovery of new clinically useful antifungal compounds.

Although a gene involved in the cellular growth 30 of *S. cerevisiae* was identified, there are no certainties that there would be a homolog in *Candida albicans* or if present that it would have the same function.

In accordance with the present invention a gene 35 was isolated, *CaKRE9*, in the yeast pathogen, *Candida albicans*, that is a functional homolog of the *S. cerevisiae KRE9* gene and which is essential for cell wall

glucan synthesis. The gene is not found in humans and when it is inactivated in *C. albicans*, the cell cannot survive when grown on glucose, thus, validating it as a wholly new target for antifungal drug discovery.

5 Using the gene of the present invention, we intend to utilize novel drug screening assays for which we possess all the genetic tools.

In accordance with the present invention there is provided an isolated DNA which codes for a gene 10 essential for cell wall glucan synthesis of *Candida albicans*, wherein the gene is referred to as *CaKRE9*, wherein the sequence of the DNA is as set forth in Fig. 1.

In accordance with the present invention there 15 is also provided an antifungal screening assay for identifying a compound which inhibits the synthesis, assembly and/or regulation of β 1,6-glucan, which comprises the steps of:

- 20 a) synthesizing β 1,6-glucans *in vitro* from activated sugar monomer/polymer and specific β 1,6-glucan synthetic proteins;
- b) subjecting step a) to a high throughput compound screen determining absence or presence of β 1,6-glucan, wherein absence of β 1,6-glucan is 25 indicative of an antifungal compound.

In accordance with the present invention there is also provided an *in vivo* antifungal screening assay for identifying compounds which inhibit the synthesis, assembly and/or regulation of β 1,6-glucan, which comprises the steps of:

- a) separately cultivating a mutant yeast strain lacking one gene for synthesis of β 1,6-glucans and a wild type yeast strain with activated sugar monomer/polymer UDP-glucose;

- 5 b) subjecting both yeast strains of step a) to the screened compound and determining if the compound selectively inhibits growth of wild type strain which is indicative of an antifungal compound.

In accordance with the present invention there is also provided an *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, which comprises the steps of:

- 10 a) obtaining a biological sample from the patient;
b) subjecting the sample to PCR using a primer pair specific for *CaKRE9* gene, wherein a presence of the gene is indicative of the presence of fungal infection.

15 In accordance with the present invention, the gene is *CaKRE9*.

In accordance with the present invention there is also provided an *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, 20 which comprises the steps of:

- 25 a) obtaining a biological sample from the patient;
b) subjecting the sample to an antibody specific for *CaKre9p* antigen, wherein a presence of the antigen is indicative of the presence of fungal infection.

In accordance with one embodiment of the present invention, the fungal infection may be caused by *Candida*.

30 In accordance with the present invention there is also provided the use of at least one of *KRE9* and *CaKre9* nucleic acid sequences and fragments thereof as a probe for the isolation of *KRE9* homologs in all fungi.

35 For the purpose of the present invention the following terms are defined below.

The term a "mutant yeast strain" is intended to mean any yeast strain lacking one gene for synthesis of β 1,6-glucan, such as *KRE9* and homologs thereof.

5 The term a "wild type yeast strain" is intended to mean any yeast strain containing the *KRE9* gene or a homolog thereof or a plasmid overexpressing the *KRE9* gene or a homolog thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 illustrates the complete nucleotide and predicted amino acid sequence of *CaKRE9* (SEQ ID NO:1-2).

15 Fig. 2 illustrates the comparison of the sequence of *Kre9p* from *Candida albicans* (SEQ ID NO:2) and *Kre9p* (SEQ ID NO:3) and *Knh1p* (SEQ ID NO:4) from *Saccharomyces cerevisiae*;

Fig. 3 illustrates the *CaKRE9*-dependent effect on the growth (A) and Killer phenotype (B) of *kre9 Δ* null mutants;

20 Fig. 4A illustrates the schematic representation of the strategy for disruption of the *Candida albicans KRE9* gene;

25 Fig. 4B illustrates the Southern blot verification of the correct integration of the *hisG-URA3-hisG* disruption module into the *CaKRE9* gene and proper *CaURA3* excision after 5-FOA treatment; and

Fig. 5 illustrates the quantification of β 1,6-Glucan levels of different *Candida albicans* strains.

30 DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, the synthesis and the assembly of the cell wall polymer β 1,6 glucan which plays a central role in the organization of the yeast cell wall and which is indispensable for cell viability were extensively studied. Although

the biochemistry of β 1,6 glucosylation is incompletely understood, a genetic analysis of genes required for 1,6 synthesis has been performed in *Saccharomyces cerevisiae*, and has identified many genes required for this 5 process. These encode products acting in the endoplasmic reticulum, the Golgi complex and at the cell surface.

In accordance with the present invention a novel gene was identified, *KRE9*, whose product is required 10 for the synthesis of β 1,6 linked glucans (Brown JL. et al. (1993) *Molecular & Cellular Biology* 13:6346-6356). *KRE9* appears to be a fungal specific gene, as it is absent from animal lineages based on data base searches 15 of the *Caenorhabditis elegans*, mouse and *Homo sapiens* genomes and it also appears to be absent from the plant, bacterial and archaebacterial lineages.

KRE9 and its homolog *KNH1*

KRE9 encodes a 30-kDa secretory pathway protein involved in the synthesis of cell wall β 1,6 glucan 20 (Brown JL. et al. (1993) *Molecular & Cellular Biology* 13:6346-6356). Disruption of *KRE9* in *S. cerevisiae* leads to serious growth impairment and an altered cell wall containing less than 20% of the wild-type amount 25 of β 1,6 glucan. Analysis of the glucan material remaining in a *kre9* null mutant indicated a polymer with a reduced average molecular mass (Brown JL. et al. (1993) *Molecular & Cellular Biology* 13:6346-6356). The *kre9* null mutants also displayed several additional 30 cell-wall-related phenotypes, including an aberrant multiple budded morphology, a mating defect, and a failure to form projections in the presence of alpha-factor. Antibodies generated against Kre9p detected an O-glycoprotein of approximately 55 to 60 kDa found in the extracellular medium of a strain overproducing

Kre9p, indicating it is normally localized at the cell surface.

In the yeast genome a *KRE9* homolog was recently found, *KNH1*, whose product, Knh1p, shares 46% overall identity with Kre9p (Dijkgraaf GJ. et al. (1996) *Yeast* 12:683-692). Disruption of the *KNH1* locus has no effect on growth, killer toxin sensitivity or β 1,6-glucan levels. Overexpression of *KNH1* suppressed the severe growth defect of a *kre9* null mutant and restored the level of alkali-insoluble β 1,6-glucan to almost wild type levels. When overproduced, Knh1p, like Kre9p, can be found in the extracellular culture medium as an O-glycoprotein, and is likely also a cell surface protein under conditions of normal expression. The disruption of both *KNH1* and *KRE9* is lethal. Transcription of *KNH1* is carbon-source and *KRE9* dependent. The severe growth defect of a *kre9Δ* null mutant observed on glucose can be partially restored when galactose becomes the major carbon source. Transcription of the *KNH1* gene is normally low in wild type cells grown on glucose but increases approximately five fold in galactose grown cells, where it partially compensates for the loss of Kre9p and allows partial suppression of the slow growth phenotype of *kre9Δ* cells. These results suggest that *KRE9* and *KNH1* are specialized *in vivo* to function under different environmental conditions (Dijkgraaf GJ. et al. (1996) *Yeast* 12:683-692).

The essential nature of the *KRE9/KNH1* gene pair, and the putative extracellular location of their gene products make these proteins a new and potentially valuable target for antifungal compounds that need not enter the fungal cell.

β 1,6-glucan in pathogenic fungi

The yeast *Saccharomyces cerevisiae*, although not a pathogen, is a proven model organism for pathogenic

fungi as it is closely related taxonomically to opportunistic pathogens like the dimorphic yeast *Candida albicans*. The composition of the cell wall of *C. albicans* resembles that of *S. cerevisiae* in containing
5 β 1,3- and β 1,6-glucans, chitin, and mannoproteins (Mio, T. et al., *J. Bacteriol.* 179:2363-2372) Analyses of the *Candida albicans* genes involved in extracellular matrix assembly are limited but indicate that the proteins responsible for synthesis of the polymers often resemble those found in the more extensively studied yeast,
10 *Saccharomyces cerevisiae*. The β 1,6 glucosylation of proteins appears to be widespread among fungal groups, and the polymer varies in abundance between fungal species. In *C. albicans* this polymer is particularly
15 abundant, comprising approximately half of the alkali insoluble glucan. Comparative studies with *C. albicans* have so far identified three genes involved in β 1,6 glucosylation based on their relatedness to those in *S. cerevisiae*, indicating that synthesis of this polymer
20 is functionally conserved and essential for the growth of *Candida albicans*.

Isolation of the CaKRE9 gene

In order to validate *KRE9* as a possible new antifungal target, we have examined if genes related to
25 *S. cerevisiae KRE9* were present in *C. albicans*. Using complementation of the *S. cerevisiae kre9* mutant phenotype as a screen, we have isolated a *C. albicans* gene that encodes a protein similar to the *S. cerevisiae KRE9* gene product.

30 CaKRE9 was identified by a plasmid shuffle approach as a gene being able to restore the slow growth of a *Saccharomyces cerevisiae kre9::HIS3* disrupted strain. A diploid strain heterozygous for a *kre9::HIS3* deletion was transformed with a centromeric
35 *LYS2*-based pRS317 vector containing a wild type copy of

the *S. cerevisiae* *KRE9* gene. Transformants were selected by prototrophic growth on minimal media, sporulated and a haploid *kre9::HIS3* strain containing a plasmid-based copy of *KRE9* was obtained by tetrad dissection and spore progeny analysis. This strain was shown to possess wild type growth and killer toxin sensitivity and was subsequently transformed with a *Candida albicans* genomic library contained within the multicopy YEpl352-plasmid harboring the *URA3* gene as a selectable marker. In order to screen for plasmids that could restore growth to a *kre9::HIS3* mutant, about 20,000 His3⁺ Lys2⁺ Ura3⁺ cells were replica plated on minimal medium containing α-amino adipate as a primary nitrogen source to select for cells that have lost the *LYS2* plasmid-based copy of *KRE9* but are still able to grow, indicating that a copy of the complementing *CaKRE9* gene could be present in such growing cells. These cells were further tested for loss of the pRS317-*KRE9* plasmid by failure to grow on medium lacking lysine. YEpl352-based *Candida albicans* genomic DNA was recovered from cells that grew in the presence of lysine but did not grow in its absence. Upon retransformation in yeast, only 2 different genomic inserts were able to partially restore growth of the *kre9::HIS3* haploid strain. DNA from both inserts were sequenced.

The *CaKRE9* gene was contained in only one of the *C. albicans* clones. Complete sequencing of the 8-kb fragment containing the *CaKRE9* gene revealed an open reading frame of 813 bp encoding a 29-kDa secretory protein of 271 amino acid residues (see Fig. 1). As is the case with Kre9p and Knh1p (Brown JL. et al. (1993) *Molecular & Cellular Biology* 13:6346-6356; Dijkgraaf GJ. et al. (1996) *Yeast* 12:683-692), the hydrophobic N-terminal region of CaKre9p comprises an eukaryotic sig-

nal sequence, with the most likely cleavage site occurring between amino acid residues 21 and 22. CaKre9p shares 43% overall identity with Kre9p and 32% with Knh1p (see Fig. 2). The amino acid residues are shown 5 in single-letter amino acid code. Sequences were aligned with gaps to maximize homology. Dots represent a perfect match between all sequences while a vertical slash indicates conservative substitution at a given position. The most conserved region between the 3 proteins encompasses a large part of the central region and most of the C-terminal portion, with the N-terminal part being largely unique to each protein. Kre9p, Knh1p and CaKre9p share a high proportion of serine and threonine residues (26%), potential sites for O-glycosylation, a modification known to occur on Kre9p and Knh1p, and characteristic of many yeast cell surface proteins. In addition, all 3 proteins have lysine and arginine rich C-termini and lack potential N-linked glycosylation sites.

20 The functional capacity of CaKre9p was assessed in *Saccharomyces cerevisiae* by measuring its ability to restore the growth and killer toxin sensitivity of a *kre9* null mutant. Firstly, the YEp352-based *Candida albicans* genomic DNA containing the *CaKRE9* gene was 25 transformed into a diploid strain of *S. cerevisiae* heterozygous for a *kre9::HIS3* deletion, sporulated and a haploid *kre9::HIS3* strain containing a plasmid-based copy of *CaKRE9* was obtained from spore progeny following tetrad dissection. As can be seen in Fig. 3A, a 30 strain harboring the *CaKRE9* gene grows at a slower rate than a wild type strain or the mutant strain harboring a copy of *KRE9* but significantly faster than the *kre9* null mutant which has a severe growth phenotype. Secondly, the haploid *kre9* strain carrying the *CaKRE9* was 35 submitted to a killer toxin sensitivity assay (Fig.

3B). K1 killer yeast strains secrete a small pore-forming toxin that requires an intact cell wall receptor for function. *KRE9* null mutations lead to a considerable decrease in the level of β 1,6-glucan disrupting the toxin receptor (Brown JL. et al. (1993) *Molecular & Cellular Biology* 13:6346-6356), leading to killer resistance and showing no killing zone in the assay. The killer phenotype of the *kre9* mutant allowed a test of possible suppression by CaKre9p. Overexpression of 10 Ca*KRE9* in the *S. cerevisiae* haploid strain carrying a disrupted copy of *KRE9* partially suppressed the killer resistance phenotype (Fig. 3B).

These results imply that Kre9p and CaKre9p both play very similar roles in β 1,6-glucan assembly in *S. cerevisiae* and *C. albicans*.

Disruption of the Ca*KRE9* gene

Experimental strategy:

The gene disruption was performed by the URA blaster protocol using the *hisG-CaURA3-hisG* module. A 20 1.6-kb DraI DNA fragment containing the Ca*KRE9* gene was subcloned from the original insert into the SmaI site and the blunted XbaI site (treated with the Klenow fragment of DNA polymerase I) of YEp352 (see Fig. 4A). Extracted genomic DNAs are from : CAI4 wild type cells 25 (lane 1), Ca*KRE9/Cakre9::hisG-URA-hisG* heterozygous mutant (lane 2), Ca*KRE9/Cakre9::hisG* heterozygous mutant obtained after 5-FOA treatment (lane 3) and Cakre9/Cakre9::*hisG-URA-hisG* homozygous null mutant which is able to grow only when galactose is used as 30 the sole source of carbon.

The Ca*KRE9* gene was disrupted by deleting a 485 bp BstXI-BamHI fragment of the open reading frame and replacing it by a 4.0 kb BglII/BamHI fragment carrying the *hisG-URA3-hisG* module from plasmid pCUB-6 (see 35 Fig. 4A). The sticky ends were enzymatically treated to

accommodate the ligation. This disruption plasmid was digested by HindIII and KpnI, precipitated with ethanol and sodium acetate and 100 µg of the 5.2 kb-disruption fragment was transformed into CAI4 *Candida albicans* 5 cells by the lithium acetate method.

Putative heterozygous disruptants were selected on minimal medium carrying glucose or galactose as carbon sources but lacking uracil. In preparation for a second round of gene disruption, the CaURA gene was 10 excised using a 5-FOA selection. The second round of transformation was performed in the same way as the primary one.

The accurate integration of the hisG-CaURA3-hisG cassette into the CaKRE9 gene and its excision from 15 genomic DNA was verified by Southern hybridization using 3 different probes:

- (1) a 405-bp fragment from *C. albicans* genomic DNA containing coding and 3' flanking sequences of CaKRE9;
- (2) a 783 bp DNA fragment obtained by PCR and covering 20 the entire CaURA3 coding region; and
- (3) a 898 bp fragment amplified by PCR that encompasses the whole of the *Salmonella typhimurium* hisG gene (see Fig. 4B).

All genomic DNAs were digested with the BamHI 25 and SalI restriction enzymes.

Results:

In the first round of transformation where transformants were selected on glucose containing plates, the Southern blotting results revealed that the 30 hisG-CaURA3-hisG module correctly integrated into the *Candida albicans* KRE9 gene (see Fig. 4). When genomic DNA of putative heterozygous CaKRE9 disruptions was digested with the SalI and BamHI restriction enzymes and probed with the CaKRE9 405-bp SalI-BstXI DNA fragment along with the hisG and the CaURA3 probes, 2 35

expected bands could be detected (see Fig. 4B, lane 2, for representative result): a 773 bp band corresponding to the wild type gene that could only be detected by the CaKRE9 probe and a 4318 bp diagnostic band, 5 revealed by all 3 probes, indicating successful disruption of one copy of the CaKRE9 gene. After removal of the CaURA3 using 5-FOA, the 773 bp wild type band could still be visualized but the disrupted band from which the CaURA3 was excised shifted to an anticipated 1428 10 bp when probed with the CaKRE9 and hisG probes but not with the CaURA3 probe (see Fig. 4B, lane 3).

In order to assess if the CaKRE9 gene is essential in *C. albicans*, a second round of disruptions was undertaken in the heterozygous strain where the CaURA3 15 gene was eliminated. However, in view of the nature of the carbon source regulation of the KRE9/KNH1 pair in *S. cerevisiae*, the second round of transformation was executed using both glucose and galactose as carbon sources. 32 Ura⁺ colonies from the glucose plated 20 transformation were analyzed by Southern blot hybridization using the 3 different probes and only yeast cells heterozygous at the CaKRE9 locus could be found. The absence of the expected homozygous double disruption among the transformants is consistent with the 25 fact that CaKRE9 is an essential gene in *C. albicans* when glucose is the sole carbon source. Demonstration of CaKRE9 as an essential gene under these conditions validates the CaKRE9 gene product as a therapeutic target in *Candida albicans*.

30 The population of transformants growing on galactose was heterogeneous with large and small sized colonies occurring. As a first assessment of a possible carbon source dependence, a total of 26 colonies of different sizes were plated from galactose to glucose. 35 Among the smaller ones, 8 did not grow on glucose, sug-

gesting that they could be homozygous disruptants. Southern blot hybridizations were performed on these 8 transformants and they were shown to be homozygous disruptants for the CaKRE9 locus: one copy corresponded to the disrupted gene in which CaURA3 has been removed (1428 bp) and the second one represented the inactivation of the remaining wild type copy by the hisG-caURA3-hisG module (4318 bp; Fig. 4B, lane 4). Thus a homozygous disruption of kRE9 in *C. albicans* is lethal when glucose constitutes the exclusive carbon source. Further, it should be appreciated that glucose is the main source of carbon of human beings.

β 1,6-glucan analysis of *C. albicans* CaKRE9 mutants

Experimental strategy:

Yeast total-cell protein extracts were prepared from exponentially growing cultures by cell lysis with glass beads. Cellular extracts were standardized for total cellular protein and equivalent amounts of protein were alkali extracted (0.75M NaOH final 1h, 75°C). The alkali soluble fractions were then spotted onto nitrocellulose and immunoblots were carried out. Briefly, blots were treated in TBST buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween™ 20, containing 5% non fat dried milk powder), and subsequently incubated with affinity purified rabbit anti- β 1,6-glucans antibodies (prepared as described Montijn, R.C. et al. (1994) *J. Biol. Chem.* 269:19338-19342) in the same buffer. After antibody binding, membranes were washed in TBST and a second antibody directed against rabbit immunoglobulins and conjugated with horseradish peroxidase, was then added. The blots were again washed and whole cell β 1,6 glucans detected using an enhanced chemiluminescence procedure.

Results

In order to directly measure the effect of inactivating CaKRE9 on β 1,6-glucan synthesis and assembly, a specific rabbit anti- β 1,6-glucan antiserum was raised against BSA-coupled pustulan (a commercially available β 1,6 glucan), affinity purified, and used to detect antigen-antibody complexes by Western blotting of total cell protein extracts of different yeast strains grown on galactose. As expected, wild type cells yielded a strong β 1,6-glucan signal (see Fig. 5). The affinity purified Ab detected about a quarter of the glucan in the *C. albicans* heterozygous Δ cakre9 whereas no β 1,6-glucan could be detected from a *C. albicans* homozygous Δ cakre9 disruptant grown on galactose (Fig. 5).

Discussion

The essential nature of the KRE9 gene in *C. albicans*, and the possible extracellular location of its gene product make this protein a new and potentially valuable target for antifungal compounds that need not enter the fungal cell. The precise role of Kre9p in β -glucan synthesis remains to be precisely determined but does not prevent the establishment of a antifungal drug screening assay

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

In vitro screening method for specific antifungal agents (enzymatic-based assay)

The primary objective is to identify novel compounds inhibiting the synthesis, assembly and/or regulation of β 1,6-glucans. This enzymatic assay would utilize some of the gene products (KRE) involved in β 1,6-glucan synthesis, including using an *in vitro* assay for CaKre9p. Using specific reagents such as an antibody to β 1,6-glucan, and a specific glucanase for

the polymer, the approach is to synthesize the polymer *in vitro* from the activated sugar monomer UDP-glucose. This task can be accomplished by existing methodologies such as the production of large amounts of each protein 5 and by the availability of genetic tools, such as the ability to delete or overexpress gene products that are involved in synthesis of this and the other major polymers. Once the assay has been established it will permit the screening of possible compounds that inhibit 10 steps in the synthesis of this essential polymer. When such inhibitors will be found, they will then be evaluated as candidates for specific antifungal agents.

The effects of such compounds on β 1,6-glucan levels may be directly measured using the anti- β 1,6-glucan antibody. This approach can be used on all type of fungi and can be adapted to a high throughput immunoassay to find β 1,6-glucan inhibitors.

EXAMPLE II

20 **In vivo screening method for specific antifungal agents
(cellular-based assay)**

Yeast strains possessing or lacking β 1,6-glucans permit a differential screen for compounds inhibiting synthesis of this cell wall polymer. Specifically, an 25 antifungal drug screen can be devised based on a whole-cell assay in which the fungal-specific CaKre9p would be targeted.

The strains that may be used in accordance with the present invention include, without limitation, any 30 yeast strain mutant for CaKRE9 and homologs thereof disrupted strain, conditional mutants, overexpression strains and suppressed disrupted strains.

Compounds can be tested for their ability to inhibit growth or kill a wild type *C. albicans* strain 35 while having no effect on a Cakre9 suppressor strain. In addition, compounds leading to hypersensitivity in a

CaKRE9 deletion will also be of value as candidate antifungal drugs. The finding of new antifungal compounds will be greatly simplified by these types of screens. The direct scoring on cells of the level of 5 efficacy of a particular compound (natural product extracts, pure chemicals...) alleviates the costly and labor intensive establishment of an *in vitro* enzymatic assay. The availability of genetic tools, such as the ability to delete or overexpress gene products that are 10 involved in synthesis of this and the other major polymers will permit the establishment of this new screening method. When such inhibitors will be found, they will then be evaluated as candidates for specific anti-fungal agents.

15

EXAMPLE III

The use of CaKRE9 in the diagnosis of fungal infection

Detection based on PCR

Candida spp. and other pathogenic fungi are traditionally identified by morphological and metabolic characteristics and often this require days to weeks to isolate on culture from a patient's sample. Identification is time-consuming and often unreliable and this impedes the selection of antimicrobial agents in cases 20 in which species identification of the organism is necessary. Moreover, culture-based diagnostic methods are not within the scope of many routine microbiology laboratories and are frequently limited to detection of pathogenic organisms in patients at an advanced stage 25 of disease or even at autopsy. The detection of disseminated Candida mycosis is an area where there is an urgency for new sophisticated techniques of identification. Polymerase Chain Reaction (PCR) based tests to establish the presence of a fungal infection are at 30 this point highly desirable for laboratory diagnosis and management of patients with serious fungal dis-

eases. The CaKRE9 gene is fungi specific and could be used to develop new diagnostic procedures of mycosis based on the PCR. Such diagnostic tests would be predicted to be highly sensitive and specific. Ultimately, simple kits permitting the diagnosis of fungal infections will be sold to hospitals and specialized clinics. Current trends in the hospital microbiology laboratories indicate that there will be a considerable future increase in use of the PCR as a diagnostic tool.

10 **Detection based on anti-CaKre9p antibodies**

CaKre9p is thought to be localized at the cell surface and as such could be detected as a circulating candidal antigen by an enzyme-linked immunoabsorbent assay (ELISA) detection kit based on antibodies directed against CaKre9p. Antibodies directed against CaKre9p could allow levels of specificity and sensitivity high enough to permit commercialization of a diagnostic kit.

20

EXAMPLE IV

The use of Kre9p in all fungi

Isolation and use of functional homologs of KRE9/CaKRE9 from all fungi. Most fungi have β 1,6-glucans and likely have KRE9 homologs in their genome. The kre9 mutant can allow isolation of similar genes by functional complementation from other pathogenic fungi as what was done to isolate CaKRE9. KRE9 could also serve as a probe to isolate by homology KRE9 homologs from other yeasts. In addition, Kre9p allows isolation of homologs in other species by the techniques of reverse genetics where antibodies raised against Kre9p could be used to screen expression libraries of pathogenic fungi for expression of KRE9 homologs that would immunologically cross react with antibodies raised against *S. cerevisiae* KRE9 and *C. albicans* CaKRE9.

These putative *KRE9* homologs in these pathogenic fungi could serve as targets for potential new antifungals.

Other methods are used to find proteins which interact with Kre9p and homologs thereof, such as two-
5 hybrid, co-immunoprecipitation and chromatography using an activated Kre9p matrix.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications
10 and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the
15 art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein said gene is referred to as *CaKRE9*, wherein the sequence of said DNA is as set forth in Fig. 1 and yeast homologs thereof.

2. An antifungal screening assay for identifying a compound which inhibits the synthesis, assembly and/or regulation of β 1,6-glucan, which comprises the steps of:

- a) synthesizing β 1,6-glucan *in vitro* from activated sugar monomer/polymer and specific β 1,6-glucan synthetic proteins;
- b) subjecting step a) to a high throughput compound screen determining concentration of β 1,6-glucan, wherein reduction in β 1,6-glucan is indicative of an antifungal compound.

3. The antifungal screening assay of claim 2, wherein said β 1,6-glucan is absent.

4. An *in vivo* antifungal screening assay for identifying compounds which inhibit the synthesis, assembly and/or regulation of β 1,6-glucans, which comprises the steps of:

- a) separately cultivating a mutant yeast strain lacking one gene for synthesis of β 1,6-glucans and a wild type yeast strain with activated sugar monomer/polymer UDP-glucose;
- b) subjecting said both yeast strains of step a) to the screened compound and determining if said compound selectively inhibits growth of wild

type strain which is indicative of an antifungal compound.

5. The method of claim 3, wherein said gene is *CaKRE9*.

6. An *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, which comprises the steps of:

- a) obtaining a biological sample from said patient;
- b) subjecting said sample to PCR using a primer pair specific for *CaKRE9* gene, wherein a presence of said gene is indicative of the presence of fungal infection.

7. The method of claim 6, wherein said fungal infection is caused by *Candida*.

8. An *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, which comprises the steps of:

- a) obtaining a biological sample from said patient;
- b) subjecting said sample to an antibody specific for *CaKre9p* antigen, wherein a presence of said antigen is indicative of the presence of fungal infection.

9. The method of claim 8, wherein said fungal infection is caused by *Candida*.

10. The use of at least one of *KRE9* and *CaKre9* nucleic acid sequences and fragments thereof as a probe for the isolation of *KRE9* homologs.

Met	Arg	Gln	Phe	Gln	Ile	Ile	Leu	Ile	Ser	Leu	Val	Val	Ser	Ile	Ile	Arg
1	ATG	AGA	CAA	TTT	CAA	ATC	ATA	TTA	ATT	TCC	CTT	GTT	TCC	ATA	ATA	AGA
Cys	Val	Val	Ala	Asp	Val	Asp	Ile	Thr	Ser	Pro	Lys	Ser	Gly	Glu	Thr	Phe
52	TGT	GTT	GTC	GCA	GAT	GTT	GAC	ATC	ACA	TCA	CCA	AAG	AGT	GGA	ACT	TTT
Ser	Gly	Ser	Ser	Gly	Ser	Ala	Ser	Ile	Lys	Ile	Thr	Trp	ASP	Asp	Ser	Asp
103	TCT	GGT	AGT	TCT	GGA	TCA	GCA	AGT	ATC	AAG	ATT	ACC	TGG	GAT	TCA	GAC
Asp	Ser	Asp	Ser	Pro	Lys	Ser	Leu	Asp	Asn	Ala	Lys	Gly	Tyr	Thr	Ile	Ser
154	GAT	TCA	GAC	TCA	CCG	AAA	TCT	TTG	GAT	AAT	GCC	AAA	GGG	TAC	ACA	ATT
Leu	Cys	Thr	Gly	Pro	Thr	Ser	Asp	Gly	Asp	Ile	Gln	Cys	Leu	Asp	Pro	Leu
205	TTA	TGT	ACT	GGA	CCT	ACT	TCA	GAT	GGG	GAT	ATC	CAG	TGT	TTG	GAT	CCA
Val	Lys	Asn	Glu	Ala	Ile	Ala	Gly	Lys	Ser	Lys	Thr	Val	Ser	Ile	Pro	Gln
256	GTC	AAG	AAC	GAA	GCT	ATT	GCA	GGT	AAA	TCT	AAA	ACA	GTT	TCT	ATT	CCC
Asn	Ser	Val	Pro	Asn	Gly	Tyr	Tyr	Phe	Gln	Ile	Tyr	Val	Thr	Phe	Thr	119
307	AAC	TCA	GTA	CCT	AAT	GGT	TAT	TAC	TAT	TTC	CAA	ATT	TAC	GTT	ACT	TTC
Asn	Gly	Gly	Thr	Thr	Ile	His	Tyr	Ser	Pro	Arg	Phe	Lys	Leu	Thr	Gly	Met
358	AAT	GGA	GGT	ACC	ACT	ATT	CAT	TAT	TCA	CCA	CGT	TTC	AAA	TTG	ACT	GGT

/Q

~~THE~~ - 1A

Ser	Gly	Pro	Thr	Ala	Thr	Leu	Asp	Val	Thr	Glu	Thr	Gly	Ser	Val	Pro	Ala	
409	TCT	GGT	CCA	ACT	GCC	ACT	TTA	GAT	GTC	ACC	GAA	ACA	GGA	TCG	GTC	CCA	GCG
ASP	Gln	Ala	Ser	Gly	Phe	Asp	Thr	Ala	Thr	Ala	Asp	Ser	Lys	Ser	Phe		170
460	GAT	CAA	GCT	TCA	GGG	TTT	GAT	ACT	GCA	ACT	ACT	GCC	GAC	TCC	AAA	TCT	TTC
Thr	Val	Pro	Tyr	Thr	Leu	Gln	Thr	Gly	Lys	Thr	Arg	Tyr	Ala	Pro	Met	Gln	187
511	ACA	GTT	CCA	TAT	ACC	CTA	CAA	ACA	GGG	AAG	ACC	AGA	TAC	GCA	ATG	CAA	
Met	Gln	Pro	Gly	Thr	Lys	Val	Thr	Ala	Thr	Thr	Trp	Ser	Met	Lys	Phe	Pro	204
562	ATG	CAA	CCA	GGT	ACC	AAA	GTG	ACT	GCT	ACA	ACC	TGG	AGT	ATG	AAG	TTC	CCA
Thr	Ser	Ala	Val	Thr	Tyr	Tyr	Ser	Thr	Lys	Ala	Gly	Thr	Pro	Asn	Val	Ala	221
613	ACT	AGT	GCT	GTT	ACT	TAC	TAC	TCA	ACA	AAG	GCT	GGC	ACA	CCA	AAT	GTG	GCC
Ser	Thr	Ile	Thr	Pro	Gly	Trp	Ser	Tyr	Thr	Ala	Glu	Ser	Ala	Val	Asn	Tyr	238
664	TCT	ACT	ATT	ACC	CCA	GGT	TGG	AGT	TAT	ACT	GCT	GAA	TCT	GCC	GTT	AAC	TAT
Ala	Ser	Val	Ala	Pro	Tyr	Pro	Thr	Tyr	Trp	Tyr	Pro	Ala	Ser	Glu	Arg	Val	255
715	GCT	AGT	GTT	GCT	CCA	TAT	CCA	ACA	TAC	TGG	TAT	CCT	GCC	AGT	GAA	CGA	GTG
Ser	Lys	Ala	Thr	Ile	Ser	Ala	Ala	Thr	Lys	Arg	Arg	Arg	Trp	Leu	Asp		271
766	AGT	AAG	GCT	ACA	ATT	AGT	GCT	GCT	ACA	AAG	AGA	AGA	TGG	TTG	GAT	TGA	

~~FIRE~~ - 18

Position	Cakre9p	Kre9p	Knh1p
10	1	1	1
20	1	1	1
30	1	1	1
40	1	1	1
50	1	1	1
60	1	1	1

CaKre9p
Kre9p
Knh1p

AKGTYTISLCTGPTSDGDIQCLDPL---VKNEAIAKGSK---TVSIPQNSVPNGYYYYFQIYVT
1STFTFSLCTGP--NNNIDCVAVIASKITPSELTQDDKVSYTAEFASTLTGNNGQQYYIQVFAQ
---YTFTLCSG--TNAMIEAMATLQT-LSASDLTDNE---FNAIIENTVGTDGVYFIQVFAQ
3/8

2A

4/8

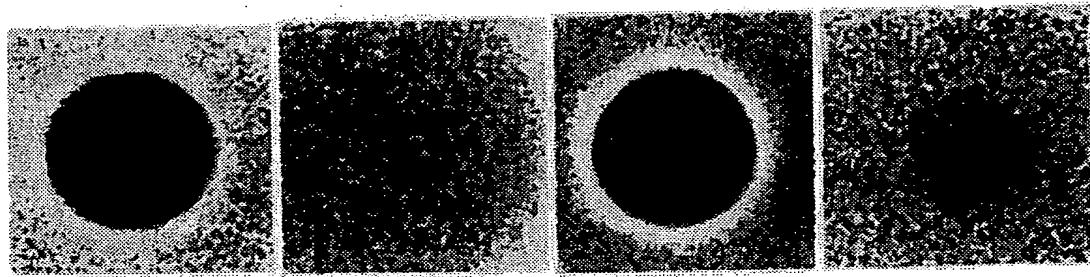
190 200 210 220 230 240 250
 | | | | | | |
 ... | ... | ... | ... | ... | ... | ... | ...
 QTGKTRYAPMQMOPGPTKVATTWSMKFPTSAVTYYSTKAGTPNVASTITPGWSYTAESAVNYA
 QTGTTSRFAPMQMOPNTKVATTWTRKEATSAVTYYSTFGSLPEQATTITPGWSYTISSGVNYA
 QTGVVKYAPMQLQPATKVATAKTwKRKYATSEVTYYTTLRNSVDQHTTVTPGWSYIITADSNYA
 Knh1p

260 270 280
 | | |
 | . | .. | . | . | . |
 SVAPYPTY - - WYPASERVS KATISAA T KRR RWLD
 TPASMP PSDNGGWYKPSKRLSLS - - ARKINMRKV
 T - APMPADNGGWYNPRKRLSLTARKVNALRHR

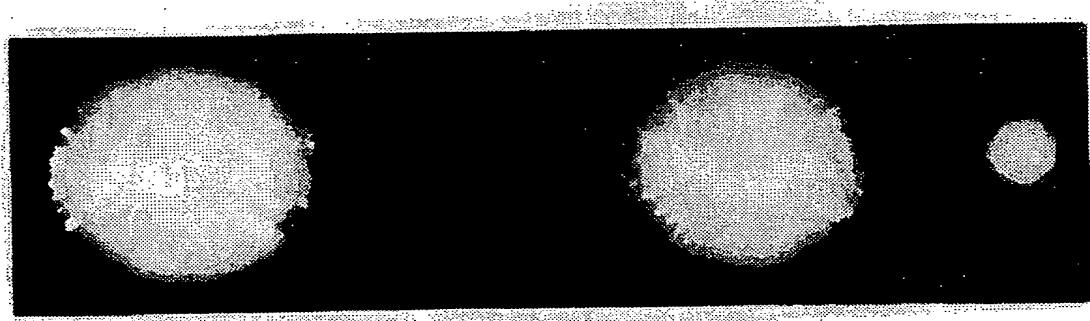
CaKre9p
 Kre9p
 Knh1p

7-11-2B

5/8



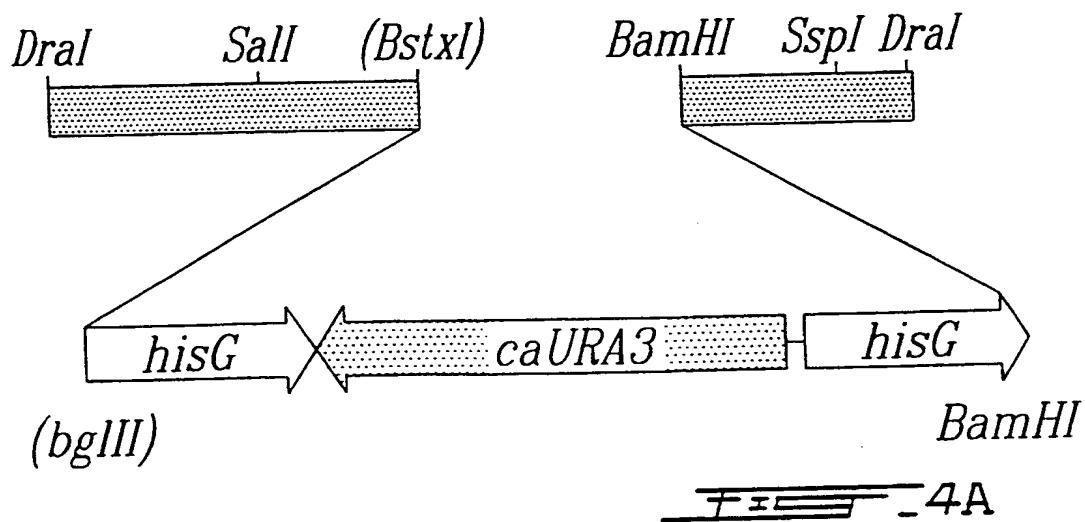
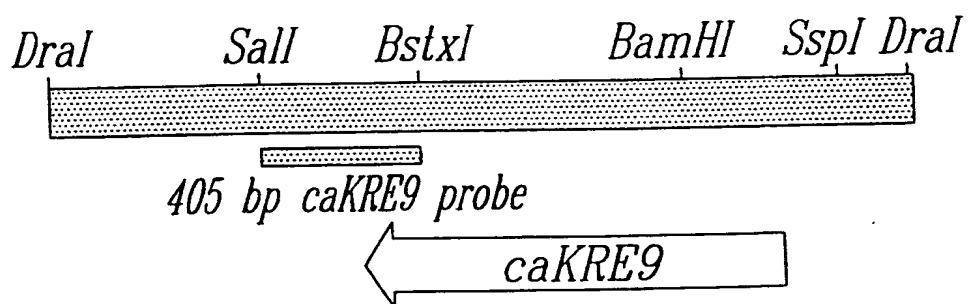
WT

 $\Delta kreg$ $\Delta kreg$
+ YEp352-KREG $\Delta kreg$
+ YEp352-CaKREG~~T~~
~~E~~
~~G~~ - 3B

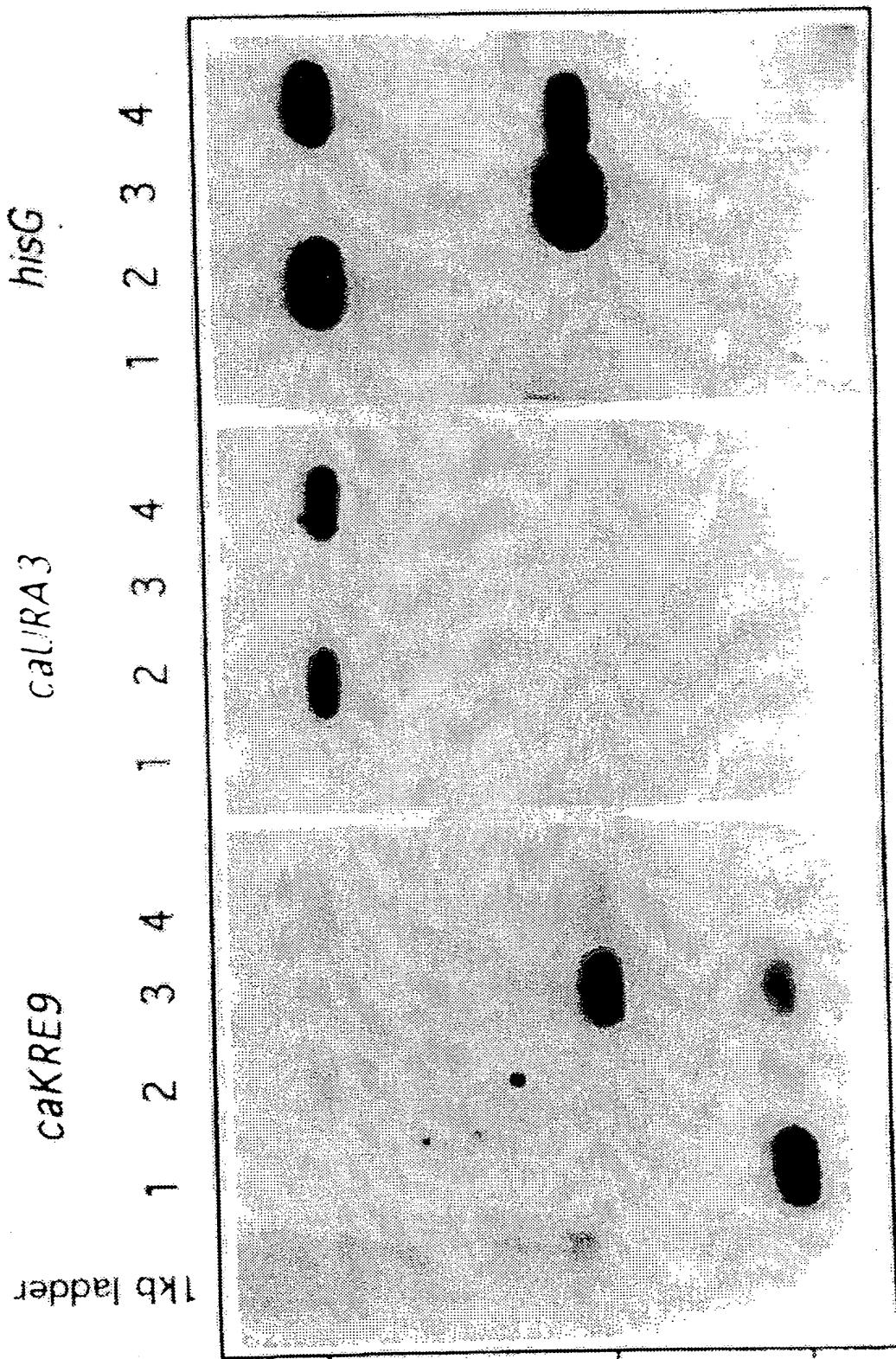
WT

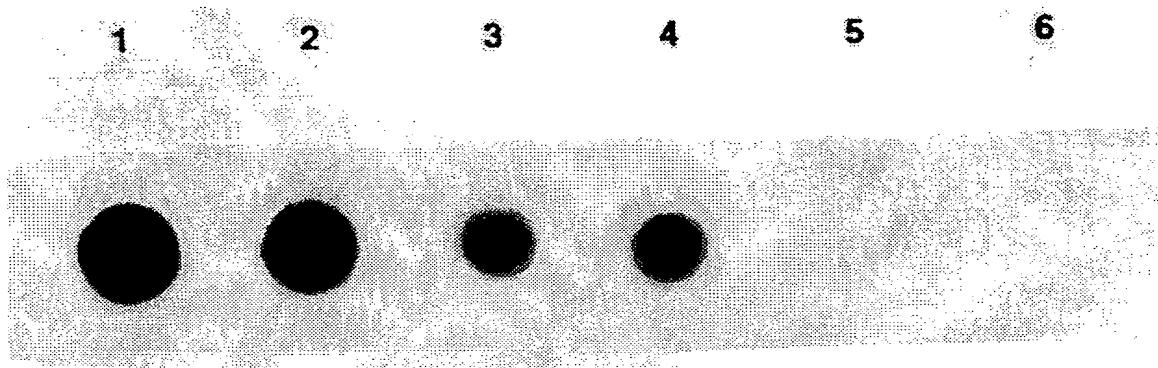
 $\Delta kreg$ $\Delta kreg$
+ YEp352-KREG $\Delta kreg$
+ YEp352-CaKREG~~T~~
~~E~~
~~G~~ - 3A

6 / 8



7/8

~~TZG~~ - 4B

8/8

1, 2: Wild type

3, 4: *CaKRE9*
*Cakre9::hisG*5, 6: *Cakre9::hisG*
Cakre9::hisG-URA3-hisG~~FIGURE 5~~

SEQUENCE LISTING

<110> MCGILL UNIVERSITY
 BUSSEY, Howard
 LUSSIER, Marc
 SDICU, Anne-Marie
 SHAHINIAN, Sarkis, Serge

<120> NEW CANDIDA ALBICANS KRE9 AND USES
 THEREOF

<130> 1770-195PCT FC/ld

<150> CA 2,218,446

<151> 1997-12-12

<160> 4

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 816

<212> DNA

<213> Artificial Sequence

<400> 1

atgagacaat ttcaaattcat attaattcc cttgttgtt ccataataag atgtgttgtt	60
gcagatgttg acatcacatc accaaagagt ggagaaactt tttctggtag ttctggatca	120
gcaagtatca agattacctg ggatgattca gacgattcag actcaccgaa atctttggat	180
aatgccaaag ggtacacaat ttctttatgt actggaccta cttcagatgg ggatatccag	240
tgtttggatc cattagtc aaacgaagct attgcaggtt aatctaaaac agtttctatt	300
ccccagaact cagtagctaa tggttattac tatttccaaa ttacgttac tttcactaat	360
ggagggtacca ctattcatta ttacccacgt ttcaaattga ctggtatgtc tggccaact	420
gccactttag atgtcaccga aacaggatcg gtgccagcggtt atcaagcttc aggatttgat	480
actgcaacta ctgcccactc caaatcttac acagttccat ataccctaca aacaggaaag	540
accagatacg caccaatgca aatgcaacca ggtaccaaag tgactgctac aacctggagt	600
atgaagtcc caactagtgc tggacttac tactcaacaa aggctggcac accaaatgtg	660
gcctctacta ttacccagg ttggagttat actgctgaat ctgccgttaa ctatgcttagt	720
gttgcctccat atccaacata ctggtatcct gccagtgaaac gagtgagtaa ggctacaatt	780
agtgcgtcta caaagagaag aagatggttt gattga	816

<210> 2

<211> 271

<212> PRT

<213> Artificial Sequence

<400> 2

Met Arg Gln Phe Gln Ile Ile Leu Ile Ser Leu Val Val Ser Ile Ile			
1	5	10	15
Arg Cys Val Val Ala Asp Val Asp Ile Thr Ser Pro Lys Ser Gly Glu			
20	25	30	
Thr Phe Ser Gly Ser Ser Gly Ser Ala Ser Ile Lys Ile Thr Trp Asp			
35	40	45	
Asp Ser Asp Asp Ser Asp Ser Pro Lys Ser Leu Asp Asn Ala Lys Gly			
50	55	60	

Tyr Thr Ile Ser Leu Cys Thr Gly Pro Thr Ser Asp Gly Asp Ile Gln
 65 70 75 80
 Cys Leu Asp Pro Leu Val Lys Asn Glu Ala Ile Ala Gly Lys Ser Lys
 85 90 95
 Thr Val Ser Ile Pro Gln Asn Ser Val Pro Asn Gly Tyr Tyr Phe
 100 105 110
 Gln Ile Tyr Val Thr Phe Thr Asn Gly Gly Thr Thr Ile His Tyr Ser
 115 120 125
 Pro Arg Phe Lys Leu Thr Gly Met Ser Gly Pro Thr Ala Thr Leu Asp
 130 135 140
 Val Thr Glu Thr Gly Ser Val Pro Ala Asp Gln Ala Ser Gly Phe Asp
 145 150 155 160
 Thr Ala Thr Thr Ala Asp Ser Lys Ser Phe Thr Val Pro Tyr Thr Leu
 165 170 175
 Gln Thr Gly Lys Thr Arg Tyr Ala Pro Met Gln Met Gln Pro Gly Thr
 180 185 190
 Lys Val Thr Ala Thr Thr Trp Ser Met Lys Phe Pro Thr Ser Ala Val
 195 200 205
 Thr Tyr Tyr Ser Thr Lys Ala Gly Thr Pro Asn Val Ala Ser Thr Ile
 210 215 220
 Thr Pro Gly Trp Ser Tyr Thr Ala Glu Ser Ala Val Asn Tyr Ala Ser
 225 230 235 240
 Val Ala Pro Tyr Pro Thr Tyr Trp Tyr Pro Ala Ser Glu Arg Val Ser
 245 250 255
 Lys Ala Thr Ile Ser Ala Ala Thr Lys Arg Arg Arg Trp Leu Asp
 260 265 270

<210> 3
 <211> 276
 <212> PRT
 <213> Artificial Sequence

<400> 3

Met Arg Leu Gln Arg Asn Ser Ile Ile Cys Ala Leu Val Phe Leu Val
 1 5 10 15
 Ser Phe Val Leu Gly Asp Val Asn Ile Val Ser Pro Ser Ser Lys Ala
 20 25 30
 Thr Phe Ser Pro Ser Gly Gly Thr Val Ser Val Pro Val Glu Trp Met
 35 40 45
 Asp Asn Gly Ala Tyr Pro Ser Leu Ser Lys Ile Ser Thr Phe Thr Phe
 50 55 60
 Ser Leu Cys Thr Gly Pro Asn Asn Ile Asp Cys Val Ala Val Leu
 65 70 75 80
 Ala Ser Lys Ile Thr Pro Ser Glu Leu Thr Gln Asp Asp Lys Val Tyr
 85 90 95
 Ser Tyr Thr Ala Glu Phe Ala Ser Thr Leu Thr Gly Asn Gly Gln Tyr
 100 105 110
 Tyr Ile Gln Val Phe Ala Gln Val Asp Gly Gln Gly Tyr Thr Ile His
 115 120 125
 Tyr Thr Pro Arg Phe Gln Leu Thr Ser Met Gly Gly Val Thr Ala Tyr
 130 135 140
 Thr Tyr Ser Ala Thr Thr Glu Pro Thr Pro Gln Thr Ser Ile Gln Thr
 145 150 155 160
 Thr Thr Thr Asn Asn Ala Gln Ala Thr Thr Ile Asp Ser Arg Ser Phe
 165 170 175
 Thr Val Pro Tyr Thr Lys Gln Thr Gly Thr Ser Arg Phe Ala Pro Met
 180 185 190

Gln Met Gln Pro Asn Thr Lys Val Thr Ala Thr Trp Thr Arg Lys
 195 200 205
 Phe Ala Thr Ser Ala Val Thr Tyr Tyr Ser Thr Phe Gly Ser Leu Pro
 210 215 220
 Glu Gln Ala Thr Thr Ile Thr Pro Gly Trp Ser Tyr Thr Ile Ser Ser
 225 230 235 240
 Gly Val Asn Tyr Ala Thr Pro Ala Ser Met Pro Ser Asp Asn Gly Gly
 245 250 255
 Trp Tyr Lys Pro Ser Lys Arg Leu Ser Leu Ser Ala Arg Lys Ile Asn
 260 265 270
 Met Arg Lys Val
 275

<210> 4
 <211> 267
 <212> PRT
 <213> Artificial Sequence

<400> 4
 Met Leu Ile Val Leu Phe Leu Thr Leu Phe Cys Ser Val Val Phe Arg
 1 5 10 15
 Thr Ala Tyr Cys Asp Val Ala Ile Val Ala Pro Glu Pro Asn Ser Val
 20 25 30
 Tyr Asp Leu Ser Gly Thr Ser Gln Ala Val Val Lys Val Lys Trp Met
 35 40 45
 His Thr Asp Asn Thr Pro Gln Glu Lys Asp Phe Val Arg Tyr Thr Phe
 50 55 60
 Thr Leu Cys Ser Gly Thr Asn Ala Met Ile Glu Ala Met Ala Thr Leu
 65 70 75 80
 Gln Thr Leu Ser Ala Ser Asp Leu Thr Asp Asn Glu Phe Asn Ala Ile
 85 90 95
 Ile Glu Asn Thr Val Gly Thr Asp Gly Val Tyr Phe Ile Gln Val Phe
 100 105 110
 Ala Gln Thr Ala Ile Gly Tyr Thr Ile His Tyr Thr Asn Arg Phe Lys
 115 120 125
 Leu Lys Gly Met Ile Gly Thr Lys Ala Ala Asn Pro Ser Met Ile Thr
 130 135 140
 Ile Ala Pro Glu Ala Gln Thr Arg Ile Thr Thr Gly Asp Val Gly Ala
 145 150 155 160
 Thr Ile Asp Ser Lys Ser Phe Thr Val Pro Tyr Asn Leu Gln Thr Gly
 165 170 175
 Val Val Lys Tyr Ala Pro Met Gln Leu Gln Pro Ala Thr Lys Val Thr
 180 185 190
 Ala Lys Thr Trp Lys Arg Lys Tyr Ala Thr Ser Glu Val Thr Tyr Tyr
 195 200 205
 Tyr Thr Leu Arg Asn Ser Val Asp Gln His Thr Thr Val Thr Pro Gly
 210 215 220
 Trp Ser Tyr Ile Ile Thr Ala Asp Ser Asn Tyr Ala Thr Ala Pro Met
 225 230 235 240
 Pro Ala Asp Asn Gly Gly Trp Tyr Asn Pro Arg Lys Arg Leu Ser Leu
 245 250 255
 Thr Ala Arg Lys Val Asn Ala Leu Arg His Arg
 260 265

THIS PAGE BLANK (USPTO)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68		A3	(11) International Publication Number: WO 99/31269
			(43) International Publication Date: 24 June 1999 (24.06.99)
<p>(21) International Application Number: PCT/CA98/01151</p> <p>(22) International Filing Date: 10 December 1998 (10.12.98)</p> <p>(30) Priority Data: 2,218,446 12 December 1997 (12.12.97) CA</p> <p>(71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): BUSSEY, Howard [CA/CA]; 325 Victoria, Westmount, Québec H3Z 2N1 (CA). LUSSIER, Marc [CA/CA]; 7790 St-Gérard, Montréal, Québec H2R 2K4 (CA). SDICU, Anne-Marie [CA/CA]; 12359 Granger, Pierrefonds, Québec H8Z 1V4 (CA). SHAHINIAN, Sarkis, Serge [CA/CA]; 5027 Christophe-Colomb, Montréal, Québec H2J 3H1 (CA).</p> <p>(74) Agents: CÔTÉ, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).</p>			
<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p> <p>(88) Date of publication of the international search report: 19 August 1999 (19.08.99)</p>			
<p>(54) Title: NEW CANDIDA ALBICANS KRE9 AND USES THEREOF</p> <p>(57) Abstract</p> <p>The present invention relates to an isolated DNA which codes for a gene essential for cell wall glucan synthesis of <i>Candida albicans</i>, wherein the gene is referred to as <i>CaKRE9</i>, wherein the sequence of the DNA is as set forth in Fig. 1. The present invention relates to antifungal <i>in vitro</i> and <i>in vivo</i> screening assays for identifying compounds which inhibit the synthesis, assembly and/or regulation of β1,6-glucan. There is also disclosed an <i>in vitro</i> method for the diagnosis of diseases caused by fungal infection in a patient.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Intern: at Application No

PCT/CA 98/01151

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C1201/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BROWN AND BUSSEY: "THE YEAST KRE9 GENE ENCODES AN O GLYCOPROTEIN INVOLVED IN CELL SURFACE BETA-GLUCAN ASSEMBLY" MOLECULAR AND CELLULAR BIOLOGY, vol. 13, no. 10, 1993, pages 6346-6356, XP002104903 see the whole document ---	1-10
Y	US 5 194 600 A (BUSSEY HOWARD ET AL) 16 March 1993 See examples III-VI see the whole document ---	1-10 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

4 June 1999

Date of mailing of the international search report

16/06/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hagenmaier, S

INTERNATIONAL SEARCH REPORT

Intern: ai Application No

PCT/CA 98/01151

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BOONE ET AL.: "ISOLATION FROM CANDIDA ALBICANS OF A FUNCTIONAL HOMOLOG OF THE SACCHAROMYCES CEREVISIAE KRE1 GENE, WHICH IS INVOLVED IN CELL WALL BETA-GLUCAN SYNTHESIS" JOURNAL OF BACTERIOLOGY, vol. 173, no. 21, 1991, pages 6859-6864, XP002104956 see the whole document</p> <p>---</p>	
A	<p>DIJKGRAAF ET A.: "THE KNH1 GENE OF SACCHAROMYCES CEREVISIAE IS A FUNCTIONAL HOMOLG OF KRE9" YEAST, vol. 12, 1996, pages 683-692, XP002104904 see the whole document</p> <p>---</p>	
A	<p>ROEMER T AND BUSSEY H: "Yeast beta-glucan synthesis: KRE6 encodes a predicted type II membrane protein required for glucan synthesis in vivo and for glucan synthase activity in vitro" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, no. 88, December 1991, pages 11295-11299 11299, XP002098181 see the whole document</p> <p>---</p>	
A	<p>WO 95 32982 A (MERCK & CO INC ;EL SHERBEINI MOHAMED (US); CLEMAS JOSEPH A (US)) 7 December 1995 see the whole document</p> <p>---</p>	
P,X	<p>LUSSIER ET AL.: "THE CANDIDA ALBICANS KRE9 GENE IS REQUIRED FOR CELL WALL BETA-1,6-GLUCAN SYNTHESIS AND IS ESSENTIAL FOR GROWTH ON GLUCOSE" PNAS, vol. 95, August 1998, pages 9825-9830, XP002104905 see the whole document</p> <p>-----</p>	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 98/01151

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US 5194600	A 16-03-1993	NONE		
WO 9532982	A 07-12-1995	US CA EP JP	5484724 A 2191067 A 0763046 A 10501408 T	16-01-1996 07-12-1995 19-03-1997 10-02-1998

THIS PAGE BLANK (USPTO)